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Nucleic Acid Polymers Inhibit Duck Hepatitis B Virus Infection *In Vitro*

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Nucleic acid polymers (NAPs) utilize the sequence-independent properties of phosphorothioate oligonucleotides (PS-ONs) to target protein interactions involved in viral replication. NAPs are broadly active against a diverse range of enveloped viruses that use type I entry mechanisms. The antiviral activity of NAPs against hepatitis B virus (HBV) infection was assessed *in vitro* in duck hepatitis B virus (DHBV)-infected primary duck hepatocytes (PDH). NAPs efficiently entered PDH in the absence of any transfection agent and displayed antiviral activity at concentrations of 0.01 to 10 μM, measured by their ability to prevent the intracellular accumulation of DHBV surface antigen, which was independent of their nucleotide sequence and was specifically dependent on phosphorothioation. Higher levels of antiviral activity were observed with NAPs 40 nucleotides in length or longer. The fully degenerate NAP (REP 2006) was active during DHBV infection or when added 12 h after infection. In contrast, an acidic-pH-sensitive NAP (REP 2031) that was broadly active against other viruses displayed antiviral activity when present during DHBV infection but no activity when added 12 h after infection, suggesting that NAPs exert their postentry effect in an acidic environment unique to DHBV infection. Both REP 2006 and REP 2031 displayed negligible cytotoxicity in PDH at concentrations of up to 10 μM, as assessed using an XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] cytotoxicity assay. The antiviral activity of NAPs against DHBV *in vitro* was strictly dependent on their amphipathic character, suggesting that NAPs interact with amphipathic target(s) that are important for DHBV entry and postentry mechanisms required for infection.

Approximately 2 billion individuals are infected with hepatitis B virus (HBV) at some point in their life. Of these, over 400 million develop chronic HBV infection, with persistent HBV DNA and HBV surface antigen (HBsAg) in serum due to continuous production of HBV antigens and HBV DNA in the liver (1). The consequences of chronic HBV infection include cirrhosis, hepatocellular carcinoma (HCC), and liver failure. These end stage disease outcomes cause over a million deaths each year (1, 2). Currently approved drugs for chronic HBV infection work well to suppress HBV replication during treatment, but virus replication generally rebounds when treatment is stopped. In addition, adverse reactions to the commonly used immunomodulators 2-alpha interferon (IFN-2α) and pegylated IFN-2α (pegIFN-2α) and the development of resistance to nucleotide/nucleoside-based viral polymerase inhibitors justify the need for research into new therapeutic agents for HBV (3, 4).

Nucleic acid polymers (NAPs) are water soluble and amphipathic in nature. These NAPs are constructed from oligonucleotides in which phosphorothioation of a nonbridging oxygen atom in the phosphodiester linkage, traditionally used as a modification to stabilize oligonucleotides against nuclease attack, is used to enhance the amphipathic properties of oligonucleotides (5). Phosphorothioation has been shown to be strictly essential for the antiviral activity of NAPs in several *in vitro* systems (6–12). The water-soluble yet amphipathic properties of NAPs are derived from inherent chemical properties of phosphorothioate oligonucleotides (PS-ONs) that are independent of the sequence of nucleotides present. NAPs have been shown to interact with structurally conserved amphipathic alpha-helical protein domains found in a variety of infectious agents, including prion proteins and type 1 viral fusion glycoproteins in human immunodeficiency virus type 1 (HIV-1) and lymphocytic choriomeningitis virus (LCMV) (9, 10, 12). In HIV-1 and LCMV, their interaction with viral fusion proteins is consistent with their ability to block viral entry. The involvement of analogous amphipathic protein structures in the surface glycoproteins of many viruses may underlie the broad-spectrum antiviral activity of NAPs against other viruses, such as herpes simplex virus 2 (HSV-2), cytomegalovirus (CMV), and hepatitis C virus (HCV) (6–8, 11), where NAPs have also been shown to act as viral entry inhibitors.

In all cases where the antiviral activity of NAPs has been examined, striking similarities in the structure-activity relationship (SAR) have been demonstrated (6–8, 10–12). The broad-spectrum antiviral activity of NAPs exhibits similar size-dependent properties in different model systems, with NAPs of <20 nucleotides (nt) in length having minimal activity, NAPs between 30 and 40 nt having increased activity with increasing length, and NAP activity becoming maximal with NAPs of >40 nt. This size-dependent target interaction is consistent with a large, sterically defined amphipathic target and was examined most extensively in HIV-1 and prion disease, in which the antiviral or antiprion activity of NAPs was shown to be directly correlated with their binding affinity to the amphipathic alpha-helices present in the fusion

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domain of HIV-1 gp41 and the conversion domains of prion protein (9, 12).

NAPs may be of therapeutic benefit to treat many viral infections, as NAPs share the well-conserved pharmacokinetic characteristics of PS-ONs in mammalian species: following administration, PS-ONs are rapidly cleared from the blood (typically less than 1 h), with significant and durable accumulations in the kidney, liver, spleen, and lung (levels of accumulation are greatest in the kidney, with decreasing but still significant amounts in liver, spleen, and lung), and up to 40% of the administered dose accumulates in the liver (13–16). Consistent with this behavior, NAPs have been shown to be active *in vivo* against virus infections with tropism in these organs: CMV, HCV, LCMV, influenza virus A, and respiratory syncytial virus (RSV) (7, 10, 11; A. Vaillant, unpublished observation).

Due to their phosphorothioated structure, NAPs are chemically analogous to sulfated polygycans. Similar to NAPs, sulfated polyglycans (for example, heparin sulfate) exhibit size- and sulfur-dependent antiviral activity against numerous enveloped viruses with type 1 entry mechanisms and prion disease (17, 18), suggesting that the antiviral targets of NAPs and sulfated polyglycans might be similar. Interestingly, sulfated polyglycans have also been shown to block the attachment/entry of HBV in a similar sulfur-dependent mechanism (19, 20), suggesting that HBV attachment and or entry might also be susceptible to inhibition by NAPs.

The current study was undertaken to test the *in vitro* antiviral activity of NAPs in duck hepatitis B virus (DHBV)-infected primary duck hepatocytes (PDH), an accepted *in vitro* model for HBV infection (21). PDH, which are prepared by collagenase perfusion of duck liver, can be readily and reproducibly infected with DHBV (22, 23). The initial steps during DHBV infection *in vitro* involve virus binding to the hepatocyte surface via the pre-S region of the large envelope protein. Duck carboxypeptidase D and glycine decarboxylase have been proposed as virus receptor molecules, and uptake of virus is thought to occur by receptor-mediated endocytosis, with cleavage of the DHBV envelope protein by a furin-like protease (24) and virus entry into early endosomes (25). Although many details of the intracellular pathways involved have not yet been determined, it has been hypothesized that the virus envelope fuses with the endosome to allow delivery of the virus nucleocapsid to the cytoplasm. In the current studies, we aimed to test the hypothesis that an attachment or fusion event in DHBV entry might be blocked by NAPs. If such activity could be discovered, the propensity of NAPs to accumulate in liver might have important therapeutic implications in the treatment of HBV infection.

MATERIALS AND METHODS

Synthesis of NAPs. All NAPs were prepared using standard solid-phase reaction conditions for the preparation of PS-ONs (Fig. 1). In the case of degenerate NAPs (REP 2006, REP 2107, REP 2086, and analogues of REP 2006 with different lengths), equal concentrations of adenosine (A), thymidine (T), guanosine (G), or cytidine (C) amidites were mixed together and used in each coupling reaction to create degenerate PS-ON of a defined length. This technique has been shown to result in pools of oligonucleotides with the same length and chemistry but with a completely degenerate sequence identity, such that no sequence-dependent functionality of any kind is present (12). Degenerate NAPs of different lengths consisted of REP 2003 (10 nt), REP 2004 (20 nt), REP 2005 (30 nt), REP 2006 (40 nt), REP 2103 (50 nt), REP 2045 (60 nt), and REP 2007 (80 nt). Oligonucleotide length was controlled by adjusting the number of coupling reactions carried out during oligonucleotide synthesis.

The 2′ O-methyl modification (found in REP 2086 and REP 2107) was incorporated by the use of 2′O-methyl modified amidites. REP 2117 (mildly hydrophobic) and REP 2118 (moderately hydrophobic) were prepared by using abasic and propane amidites, respectively. The identity of all species was confirmed by liquid chromatography-mass spectrometry (LC-MS) (data not shown). NAPs were prepared either as ammonium salts by desalting over Sephadex 250 after cleavage from the column with NH_4OH or as sodium salts by salt exchange in 3 M NaCl overnight at room temperature, followed by desalting by ultrahigh pressure filtration with water for injection. 5′ Cy3-labeled REP 2006 was prepared using a Cy3 amidite (10-5913-95; Glen Research, VA, USA). A NAP whose amphipathic activity is neutralized at acidic pH (REP 2031) was prepared by synthesizing a 40-nt PS-ON comprised only of cytosines [poly(C)] (26–28). The chemical properties of the various NAPs are described in Table 1.

Preparation of PDH. Pekin Aylesbury ducks (*Anas platyrhynchos*) were obtained at day 1 posthatch from a commercial poultry supplier. All ducks were held at the animal house facilities in SA Pathology. Animal handling protocols and standard operating procedures were approved by the Animal Ethics Committees of the SA Pathology/Central Health Network and the University of Adelaide.

PDH were prepared according to a published procedure (22). PDH were seeded at 2×10^5/well directly into 24-well tissue culture plates (Falcon, USA). PDH were seeded directly onto the plastic tissue culture plate (without coverslips) and attached to produce a confluent monolayer. PDH were initially maintained in Leibovitz's L-15 growth medium containing 5% fetal calf serum (FCS) at 37°C without CO_2. Infection was performed 2 days after plating. After infection, PDH were maintained in Leibovitz's L-15 medium without 5% FCS.

Infection of PDH. Infection of PDH was achieved using 250 viral genome equivalents of DHBV per cell and was done at 37°C. DHBV was present in a pool of infected duck sera that contained 5×10^9 viral genome equivalents of DHBV per ml, as assessed by quantitative PCR (qPCR) (29, 30). Different conditions were used to test the antiviral activity of NAPs: (i) DHBV inoculum was pretreated with prewarmed individual NAPs for 1 h at 37°C in L-15 medium before being used to infect the PDH; (ii) the PDH were pretreated with individual NAPs for 60 min at 37°C followed by washout prior to DHBV infection; and (iii) NAPs (prewarmed to 37°C) were added to the PDH at 12 h following DHBV infection. In all cases, the NAP concentrations used varied from 0.01 to 10 μM. Fresh NAPs were added during each medium change, which was done every second day. Cultures of DHBV-infected and control PDH were harvested on day 7 following DHBV infection for confocal microscopy.

Processing of PDH for confocal microscopy. At the time of harvest, the cell culture medium was removed from each well by gentle suction. The PDH were then washed with phosphate-buffered saline (PBS) at room temperature, followed by a second wash with PBS at 4°C. Finally, the PDH were fixed with ethanol acetic acid (EAA; 95:5) at 4°C. The initial EAA solution was removed by suction and replaced with fresh EAA at 4°C. After 5 min, the EAA was removed. Plates were then sealed in plastic wrap and stored at −20°C prior to immunofluorescence analysis. Immunofluorescence signal was captured in the fixed cell monolayer in single optical slices taken at the level of the nucleus.

Detection of cytoplasmic DHBsAg. DHBV surface antigen (DHBsAg) present in the cytoplasm of DHBV-infected PDH was detected using anti-DHBV pre-S monoclonal antibodies (23) and secondary goat anti-mouse polyclonal antibodies labeled with Alexa Fluor 488 (Molecular Probes, Australia). Both primary and secondary antibodies were diluted 1/200 in 1/50 normal duck serum (NDS) in PBS, and infected hepatocytes (DHBsAg positive) were detected by confocal immunofluorescence microscopy at a wavelength of 488 nm. The percentage of DHBsAg-positive PDH was determined by manual cell counts of cells judged to have positive immunofluorescence averaged over four or five

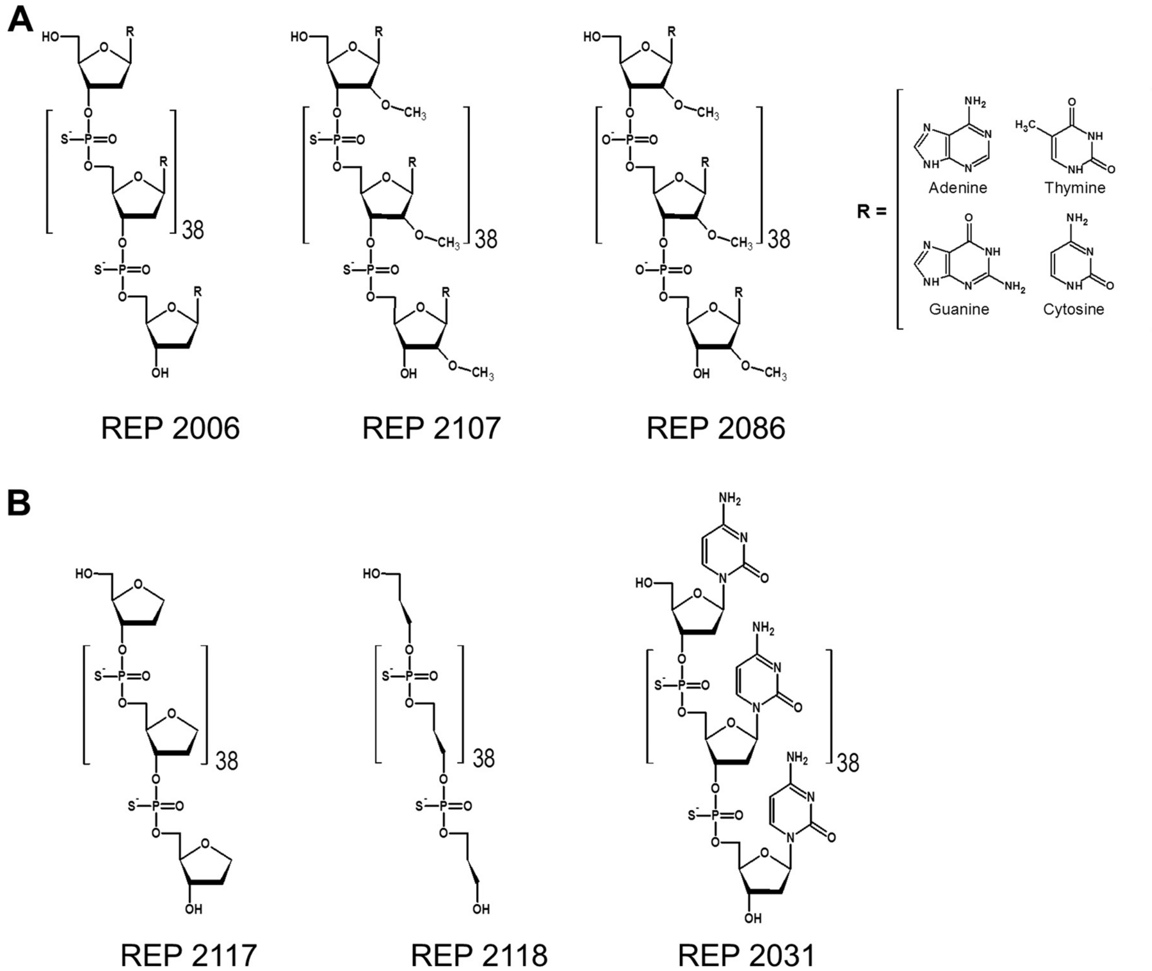

FIG 1 Structures of various NAPs tested for antiviral activity against DHBV infection in PDH and the role of the chemistry of NAPs in eliciting the antiviral activity. (A) Degenerate NAPs tested for antiviral activity. (B) NAP analogs with a defined chemistry (REP 2117 and REP 2118) or with a defined poly(C) sequence (REP 2031) tested for antiviral activity.

20× microscope fields and expressed as a percentage of total PDH per field.

Intracellular localization of Cy3-labeled REP 2006 in PDH. Cy3-labeled REP 2006 (1 μM) was prepared as described above and added to cultures of uninfected PDH in the absence of any transfection agent. Fresh Cy3-labeled REP 2006 was added during each medium change, which was done every second day. The intracellular localization of Cy3-labeled REP 2006 within PDH was observed on days 1, 4, and 7 after the start of exposure, by washing with PBS and fixation with EAA followed by confocal fluorescence microscopy at a wavelength of 585 nm as described above.

TABLE 1 Chemical properties of various nucleic acid polymers (NAPs)

NAP	Size (nt)	Bases	Chemistry
REP 2003	10	Degenerate[a]	Amphipathic (PS[b])
REP 2004	20	Degenerate	Amphipathic (PS)
REP 2005	30	Degenerate	Amphipathic (PS)
REP 2006	40	Degenerate	Amphipathic (PS)
REP 2103	50	Degenerate	Amphipathic (PS)
REP 2045	60	Degenerate	Amphipathic (PS)
REP 2007	80	Degenerate	Amphipathic (PS)
REP 2086	40	Degenerate	Hydrophilic (2′OMe[c])
REP 2031	40	Poly(C)	Nonamphipathic at acidic pH (PS)
REP 2107	40	Degenerate	Amphipathic (PS + 2′OMe)
REP 2117	40	Abasic	Mildly hydrophobic (PS)
REP 2118	40	Propane	Moderately hydrophobic (PS)

[a] Each nucleotide position is either A, G, T, or C in random fashion; thus, no sequence-specific identity is present (12).
[b] PS, phosphorothioated.
[c] 2′OMe, 2′O methylated.

Cytotoxicity assay to test for cytotoxic effects of NAP treatment of PDH. A cytotoxicity assay was performed by seeding 96-well plates (Falcon, USA) with 2×10^4 PDH per well in L-15 growth medium with 10% FCS. Stocks (10 μM) of REP 2006, REP 2031, and REP 2086 were prepared in L-15 with 10% FCS and diluted from 10 to 0.001 μM in L-15 with 10% FCS. The highest concentration of NAP (10 μM) was applied to multiples of eight wells, and all other concentrations (1.0 to 0.001 μM) were applied to multiples of four wells. Fresh REP 2006, REP 2031, and REP 2086 were added to the plate every other day. The PDH were examined each day for 7 days for any visible changes using an inverted phase-contrast microscope (Olympus CX31). On day 7, 50 μl of 1 mg/ml of tetrazolium salt (XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide]; Polysciences, Warrington, PA) was diluted in 20 ml of RPMI without phenol red (Gibco, USA). Next, 0.15 μl of 1.55-mg/ml phenazine methosulfate (PMS; Sigma Chemicals, catalogue no. P9625-1G) previously dissolved in PBS was added to the XTT to make the XTT-PMS solution. Two hundred microliters of the XTT-PMS solution was added to each treated and control well, and the plate was incubated for 4 h

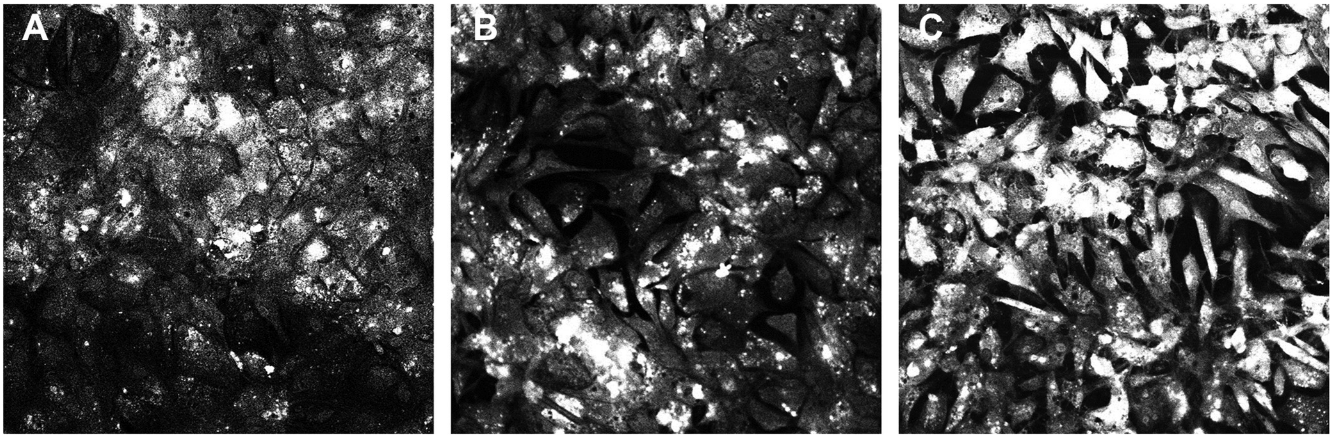

FIG 2 Intracellular localization of Cy3-labeled REP 2006 by PDH. PDH were treated with 1.0 μM Cy3-labeled REP 2006 and then fixed on days 1 (A), 4 (B), and 7 (C) with EAA, as described in Materials and Methods. Cy3-labeled REP 2006, visualized by confocal fluorescence microscopy (emission at 585 nm), was distributed diffusely in the cytoplasm and nuclei of the PDH.

at 37°C. After thorough mixing in the plate reader (Spectra Max M2; Molecular Devices, Millennium Science, USA), the absorbance at 450 nm was measured. XTT salt was reduced to an orange-colored product, XTT-formazan. This reaction occurs if there are healthy cells in the wells, as mitochondrial dehydrogenase present in viable cells metabolically reduces the XTT to a water-soluble formazan product (31).

Statistical analysis. Data were analyzed using Student's *t* test. Differences were considered to be statistically significant when the *P* values were $<$0.05.

RESULTS

***In vitro* assessment of NAPs against DHBV infection.** Previous work with NAPs using cell culture systems has shown that their antiviral activity can be achieved in the absence of mediators of transfection. Therefore, a preliminary experiment was conducted with the Cy3-labeled, degenerate NAP REP 2006 to examine the abilities of NAPs to bind to and enter uninfected PDH, in the absence of any transfection agent. REP 2006 is the prototypical antiviral NAP compound that preserves all the chemical features of NAPs (amphipathic character and optimum length of 40 nt) in the absence of any sequence identity due to its degenerate nature (Fig. 1 and Table 1). As such, REP 2006 can be used to model the cellular interactions of all NAPs with similar chemical features, regardless of sequence composition. Cy3-REP 2006 was added to cultures of uninfected PDH, and its localization within cells was observed by confocal fluorescence microscopy on days 1, 4, and 7 posttreatment. Cy3-REP 2006 was added during each medium change. Thus, PDH that were fixed on day 1 received Cy3-REP 2006 treatment once, PDH that were fixed on day 4 received Cy3-REP 2006 treatment twice, and PDH that were fixed on day 7 received Cy3-REP 2006 treatment three times. As shown in Fig. 2, Cy3-REP 2006 entered into PDH and had a diffuse cytoplasmic accumulation with more-pronounced staining developing in the perinuclear and nuclear areas with increasing NAP exposure.

A preliminary *in vitro* efficacy experiment was conducted using the prototypical amphipathic degenerate NAP REP 2006 and the nonamphipathic degenerate NAP REP 2086, both at 10 μM (Table 1; Fig. 3). NAPs were added to PDH during infection with 250 viral genome equivalents of DHBV per PDH, and the extent of infection was assessed 7 days later by confocal immunofluorescence microscopy for DHBsAg. Treatment with REP 2006 resulted in negligible DHBsAg detection, indicating antiviral activity against DHBV infection (Fig. 3B) compared to DHBV-infected and untreated control PDH (Fig. 3A). In contrast, the nonamphipathic NAP REP 2086 showed no detectable reduction in DHBsAg detection in DHBV-infected PDH (Fig. 3C) compared to the DHBV-infected and untreated control PDH, indicating a lack of antiviral activity (Fig. 3A).

To assess the stage of DHBV infection at which the NAPs were exerting their antiviral effect, different treatment paradigms were used: (i) the DHBV inoculum was pretreated with NAPs before being used to infect the PDH; (ii) the PDH were pretreated with individual NAPs for 1 h followed by a washout prior to being infected with DHBV; (iii) the NAPs were added to the PDH 12 h following DHBV infection (Table 2). The NAPs used for this experiment were REP 2006 and the low-pH-sensitive NAP, REP 2031, at concentrations from 0.01 to 1.0 μM.

When used to pretreat the DHBV inoculum prior to infection, both REP 2006 and REP 2031 reduced the number of DHBsAg-positive PDH at day 7 after infection; however, REP 2006 appeared to have better activity than REP 2031 and the antiviral effect with REP 2031 was reduced at 1.0 μM, the highest concentration tested. Pretreatment of the PDH prior to DHBV infection, with both REP 2006 and REP 2031, also reduced the number of DHBsAg-positive PDH at day 7 after infection, and again REP 2006 was marginally more active than REP 2031. When REP 2006 was added 12 h after DHBV infection, treatment again reduced the number of DHBsAg-positive PDH at day 7 after infection. In contrast, REP 2031 displayed little or no antiviral activity and did not reduce the percentage of DHBsAg-positive PDH (Table 2). The percentage of DHBsAg-positive PDH was higher at all concentrations of REP 2031 added postinfection than in untreated, DHBV-infected control PDH. While these results suggest that REP 2031 might enhance DHBV infection *in vitro*, this activity was not evident *in vivo* in DHBV-infected ducks (32).

To determine if NAPs had the same size-dependent antiviral activity as observed in other viruses, degenerate NAPs with same chemical properties as REP 2006 but of different lengths were tested at a single concentration (1.0 μM) for their antiviral activity when added during DHBV infection of PDH. This concentration was used to more easily reveal any residual antiviral activity of any

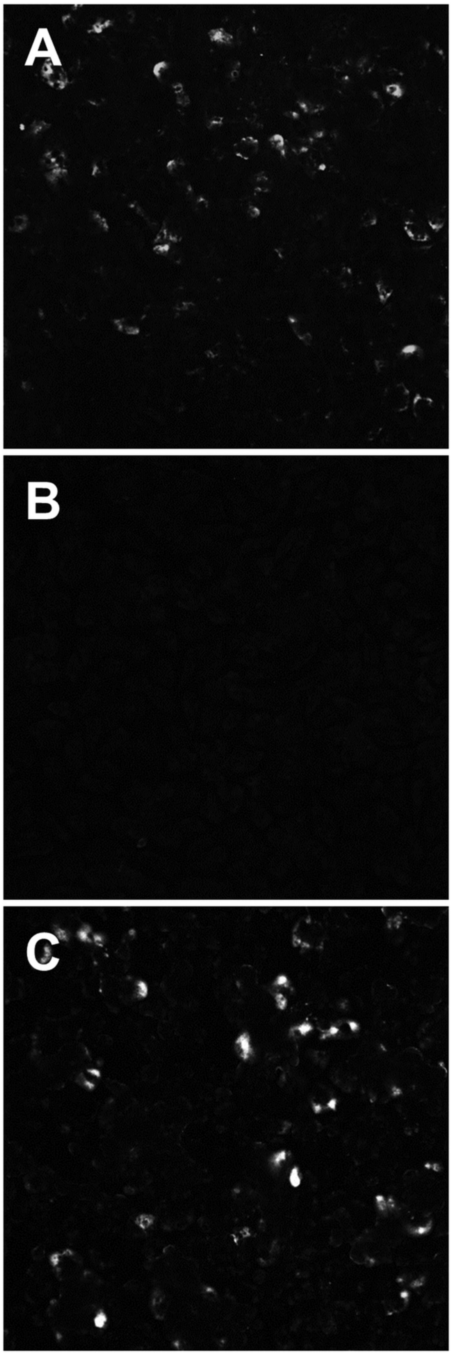

FIG 3 Detection of DHBsAg-positive PDH infected with 250 virus genome equivalents of DHBV per cell and simultaneously treated with NAPs. PDH were treated during infection with 10 μM REP 2006 (B) or REP 2086 (C). PDH were fixed on day 7 postinfection with ethanol acetic acid (EAA). DHBsAg-positive hepatocytes were visualized by confocal fluorescence microscopy as described in Materials and Methods. DHBsAg-positive PDH were detected in untreated DHBV-infected cells (A) and in infected, REP 2086-treated cells (C), while no DHBsAg-positive PDH were detected following treatment with REP 2006 (B).

of the degenerate NAPs. These degenerate NAPs were 10 to 80 nt in length (Table 1). The ability of NAPs $<$40 nt in length to reduce the percentage of DHBsAg-positive PDH was minor or negligible compared to NAPs $\geq$40 nt in length (Table 3).

The antiviral activity of NAPs against other viruses has also been shown to be strictly dependent on both their hydrophilic and hydrophobic properties. To assess the importance of these chemical features for the antiviral activity of NAPs against DHBV, several NAP analogues of the same length but with various chemical properties (Table 1) were used. These included REP 2006, REP 2086, REP 2107, REP 2117, and REP 2118 tested at a single concentration of 10 μM for their antiviral activity when added during DHBV infection of PDH (Table 3). In the presence of amphipathic NAPs (REP 2006 and REP 2107), no DHBsAg-positive PDH were observed (Table 3). NAPs that were either hydrophilic (REP 2086) or hydrophobic (REP 2118) in nature appeared to have no effect on the percentage of DHBsAg-positive PDH compared to untreated but DHBV-infected control PDH. REP 2117 (mildly hydrophobic) displayed some reduction in DHBsAg-positive PDH, but this was not as active as the amphipathic NAPs REP 2006 and REP 2107 (Table 3).

Lack of toxicity of NAPs. To assess the cytotoxicity of NAPs, an XTT-formazan cytotoxicity assay was performed on NAP-treated PDH. Regular daily microscopic examination of the REP 2006-, REP 2031-, and REP 2086-treated PDH cultures showed no detectable effects on cellular morphology (data not shown). Using the more sensitive XTT-formazan assay, treatment of PDH with REP 2006 or REP 2031 showed no significant alterations in optical density (OD) readings, while treatment with the inactive, non-phosphorothioated NAP REP 2086 resulted in increased OD readings *in vitro* (Fig. 4). These increased OD readings are likely to be an artifact of the conditions used for this *in vitro* assay, as REP 2086 was not toxic *in vitro* or *in vivo* in other studies (9, 10) and a NAP with the same chemical modifications as REP 2086 but with a defined sequence was also well tolerated *in vivo* (11).

DISCUSSION

REP 2006 was observed to readily accumulate in the cytoplasm of PDH in the absence of any transfection agent, strongly suggesting a conserved, transfection-independent entry mechanism for all amphipathic NAPs. NAPs with either a degenerate (REP 2006) or poly(C) (REP 2031) sequence displayed antiviral activity against DHBV infection of PDH *in vitro*, demonstrating that this antiviral activity was not dependent on the nucleotide sequence of the NAP. The antiviral activity of NAPs in DHBV infection *in vitro* was also shown to be size dependent, with NAPs $\geq$40 nt in length displaying the highest activity. Additionally, this antiviral activity was strictly dependent on the presence of hydrophobic and hydrophilic properties inherent in PS-ONs. REP 2086 is stabilized by the 2′O-methylation of each of its ribose moieties, this modification does not confer the amphipathic property that phosphorothioation does, and REP 2086 was not effective against DHBV infection. Moreover, phosphorothioated NAP derivatives lacking the base (REP 2117) or base and ribose structures (REP 2118) are stable but have a more pronounced hydrophobic character and also displayed little or no antiviral activity against DHBV infection *in vitro*.

When either REP 2006 or REP 2031 was added to the inoculum or to the PDH prior to infection, both displayed antiviral activity. However, when added 12 h following DHBV infection, REP 2006 was active but REP 2031 was not. The amphipathic character of REP 2031 is neutralized at acidic pH due to its homopyrimidine [poly(C)] content (26–28), and its lack of postentry antiviral activity suggests that the postentry antiviral effect of NAPs is occur-

TABLE 2 Effect of time of addition of NAPs on DHBV infection of PDH

NAP	Infection and treatment[a]	Concn of NAP (μM)	% DHBsAg-positive PDH[b] (mean ± SD)
REP 2006	DHBV inoculum pretreated with NAP prior to infection	0	12.18 ± 1.48
		0.01	0.62 ± 0.86[c]
		0.1	0 ± 0[c]
		1.0	0 ± 0[c]
	PDH pretreated with NAP followed by washout prior to DHBV infection	0	10.00 ± 2.92
		0.01	0 ± 0[c]
		0.1	0 ± 0[c]
		1.0	0 ± 0[c]
	NAP added 12 h following DHBV infection	0	11.56 ± 1.92
		0.01	3.43 ± 4.21[c]
		0.1	2.81 ± 0.83[c]
		1.0	1.88 ± 1.11[c]
REP 2031	DHBV inoculum pretreated with NAP prior to infection	0	8.43 ± 1.48
		0.01	2.5 ± 1.58[c]
		0.1	3.12 ± 1.80[c]
		1.0	6.56 ± 1.48
	PDH pretreated with NAP followed by washout prior to DHBV infection	0	13.43 ± 5.02
		0.01	1.56 ± 1.30[c]
		0.1	0.31 ± 0.43[c]
		1.0	0.44 ± 0.83[c]
	NAP added 12 h following DHBV infection	0	25.31 ± 9.36
		0.01	52.18 ± 3.03
		0.1	39.06 ± 2.59
		1.0	26.87 ± 1.12

[a] PDH were infected with 250 viral genome equivalents of DHBV per cell. NAPs (0 to 1.0 μM) were added at the times indicated and at each medium change.
[b] PDH were fixed with EAA on day 7 after DHBV infection and analyzed by confocal immunofluorescence for DHBsAg. A value of 0 means that no detectable DHBsAg-positive hepatocytes were observed.
[c] Statistically significant reduction in percentage of DHBsAg-positive PDH compared to untreated, DHBV-infected control ($P < 0.05$) as determined by the Student *t* test.

ring intracellularly in an acidic environment. The restriction of REP 2031 to entry activity when REP 2006 is active both during and after infection suggests that NAPs exert their antiviral activity *in vitro* against DHBV both by inhibiting the entry of DHBV and by a postentry mechanism required for DHBV infection and/or replication. In these experiments, the percentage of DHBsAg-positive hepatocytes was elevated in REP 2031-treated cells compared to control PDH, suggesting some enhancement of DHBV infection by REP 2031 *in vitro*. However, this enhancement of infection in the presence of REP 2031 was observed only postentry and,

TABLE 3 The antiviral activity of NAPs against DHBV infection in PDH was dependent on their length and amphipathic nature

Structural feature tested	NAP[a]	NAP length (nt)	Chemical property	% DHBsAg-positive PDH[b] (mean ± SD)
Length versus antiviral activity (tested at 1 μM)	Untreated			23.56 ± 5.62
	REP 2003	10	Amphipathic	11.56 ± 3.32
	REP 2004	20	Amphipathic	21.78 ± 2.50
	REP 2005	30	Amphipathic	22.22 ± 2.65
	REP 2006	40	Amphipathic	0 ± 0[c]
	REP 2103	50	Amphipathic	7.56 ± 4.25[c]
	REP 2045	60	Amphipathic	5.78 ± 3.25c
	REP 2007	80	Amphipathic	0 ± 0[c]
Amphipathicity versus antiviral activity (tested at 10 μM)	Untreated			15.56 ± 1.73
	REP 2006	40	Amphipathic	0 ± 0[c]
	REP 2086	40	Nonamphipathic	12.00 ± 2.75
	REP 2107	40	Amphipathic	0 ± 0[c]
	REP 2117	Equivalent to 40	Mildly amphipathic	1.78 ± 0.82[c]
	REP 2118	Equivalent to 40	Nonamphipathic	9.33 ± 1.89

[a] PDH were infected with 250 virus genome equivalents of DHBV per cell. NAPs were added during DHBV infection and at each medium change.
[b] PDH were fixed with EAA on day 7 after DHBV infection and analyzed by confocal immunofluorescence for DHBsAg. A value of 0 means that no detectable DHBsAg-positive hepatocytes were observed.
[c] Statistically significant reduction in the percentage of DHBsAg-positive PDH compared to untreated, DHBV-infected control ($P < 0.05$).

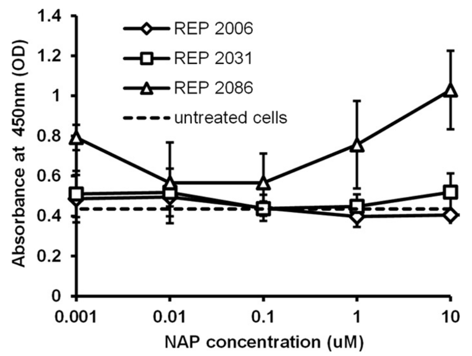

FIG 4 Cytotoxic effect of treatment of PDH with REP 2006, REP 2031, and REP 2086. PDH were treated with the NAPs REP 2006, REP 2031, and REP 2086 (0.001 to 10 μM) for 7 days before addition of XTT as described in Materials and Methods. OD values were reflective of the number of viable cells present to convert the XTT salt to an orange-colored product, XTT-formazan. OD was measured at 450 nm, and plots are mean ODs ± standard deviations (SD) from 4 wells.

given the fact that NAPs readily enter PDH, is inconsistent with the antiviral activity observed when REP 2031 was added during infection. Moreover, such enhanced antiviral activity with REP 2031 was not apparent *in vivo* in DHBV-infected ducks (32). The lack of postentry activity with REP 2031 when added before infection may explain its marginally weaker antiviral activity compared to REP 2006 (especially at lower concentrations), as REP 2031, unlike REP 2006, does not contain any postentry activity which would persist throughout the course of the infection. Thus, while subtle differences between the entry-inhibitory activity of REP 2006 and REP 2031 cannot be clarified based on the results presented here, it is clear that both these NAPs possess significant entry-inhibitory activity.

The SAR of NAPs in DHBV infection is highly congruent to that observed in other hepatotropic viruses (CMV and HCV) (7, 8, 11) and type 1 viruses (HSV-1 and -2, LCMV, and HIV-1) (6, 10, 12). In each of these previously reported cases, this unique SAR defines the antiviral target in each virus as a large amphipathic protein domain of similar size that functions during viral entry. The results from these *in vitro* investigations appear to indicate that an antiviral target with similar amphipathic functionality is also important during the DHBV life cycle. NAPs are chemically analogous to sulfated polyglycans like heparin sulfate, which has been shown to be involved in the initial steps of virus binding to the hepatocyte surface via interaction with the pre-S1 domain of the large HBsAg protein (20). Curiously, highly sulfated polyglycans like dextran sulfate and heparin sulfate (which have significant amphipathic character) were active in blocking HBV entry into hepatocytes, while poorly sulfated polyglycans like chondroitin sulfate (which is weakly amphipathic) were not active in blocking HBV entry (19, 20). This requirement for amphipathic properties for entry inhibition is similar for both NAPs and sulfated polyglycans not only in HBV but other enveloped viruses as well (18) and suggests that NAPs may block the binding of pre-S1 binding to heparin sulfate proteoglycans on the cell surface in a fashion similar to that described for sulfated glycans (19, 20). However, the nature of the postentry antiviral mechanism has yet to be elucidated and bears further examination, as it suggests the potential for a novel therapeutic effect against established HBV infection.

Assessment of cytotoxicity demonstrated that the amphipathic NAPs REP 2006 and REP 2031 had no cytotoxic effect in PDH up to 10 μM. At all concentrations, exposure of PDH to REP 2086 resulted in elevated OD_{450} readings, but the cause of this is unclear. *In vitro* toxicity with REP 2086 in other cellular models using alamarBlue as the endpoint measure for cytotoxicity has not been observed (A. Vaillant, unpublished observation), and all NAPs tested (including REP 2006 and REP 2031) had no effect on gross cellular morphology. While the 50% effective concentrations (EC_{50}s) of REP 2006 and REP 2031 were not specifically established, they appear to be <0.01 μM when added before infection (Table 2). Given that Both REP 2006 and REP 2031 displayed no significant cytotoxicity (as measured by the XTT assay) at 10 μM, the therapeutic index for these compounds is likely to be quite large. This is consistent with the generally well-tolerated nature of NAPs (including those with the same chemistry as REP 2086) *in vitro* and *in vivo* (6, 7, 9–11).

As PS-ONs, NAPs have important advantages over sulfated polyglycans like heparin sulfate. NAPs do not induce chronic anticoagulation like sulfated glycans do. Additionally, NAPs have the propensity to accumulate in the liver (13–16) with low systemic exposure, which results in additional tolerability advantages. Importantly, in this study, NAPs were effective in blocking DHBV entry *in vitro* at concentrations 10- to 100-fold lower than reported for heparin sulfate inhibition of HBV infection (19, 20), suggesting that NAPs have a much higher specific activity for targeting DHBV (and HBV) entry than sulfated polyglycans. Preliminary studies on the prophylactic activity NAPs against DHBV infection *in vivo* are presented in our accompanying paper (32).

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